# A Broad Host Range Tailocin from *Burkholderia cenocepacia*

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Running Head: Tailocin from *Burkholderia cenocepacia*

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## ABSTRACT

The *Burkholderia cepacia* complex (Bcc) consists of 20 closely related gram-negative bacterial species that are significant pathogens for persons with cystic fibrosis (CF). Some Bcc strains are highly transmissible and resistant to multiple antibiotics, making infection difficult to treat. A tailocin (phage-tail-like bacteriocin), designated BceTMilo, with broad host range against members of the Bcc was identified in *Burkholderia cenocepacia* strain BC0425. Sixty-eight percent of Bcc representing 10 species and 90% of non-Bcc *Burkholderia* strains tested were sensitive to BceTMilo. BceTMilo also showed killing activity against *Pseudomonas aeruginosa* PAO1 and derivatives. Liquid chromatography-mass spectrometry analysis of the major BceTMilo proteins was used to identify a 23 kb tailocin locus in a draft BC0425 genome. The BceTMilo locus was syntenic and highly similar to a 24.6 kb region on chromosome 1 of *B. cenocepacia* J2315 (BCAL0081 to BCAL0107). A close relationship and synteny were observed between BceTMilo and *Burkholderia* phage KL3, and by extension to the paradigm temperate myophage P2. Deletion mutants in the gene cluster encoding enzymes for biosynthesis of lipopolysaccharide (LPS) in the indicator strain *B. cenocepacia* K56-2 conferred resistance to BceTMilo. Analysis of the defined mutants in LPS biosynthetic genes indicated that α-D-glucose residue in the core oligosaccharide is the receptor for BceTMilo.

## IMPORTANCE

BceTMilo, presented in this study, is a broad host range tailocin active against *Burkholderia spp*. As such, BceTMilo and related or modified tailocins, have potential as bactericidal therapeutic agents against plant and human pathogenic *Burkholderia*.

## INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) is a group of gram-negative bacterial species, most of which are found in the natural environment and are not pathogenic to healthy humans. Some of these species, however, are opportunistic pathogens and pose serious risks to the health of persons with cystic fibrosis (CF) (1, 2). Among Bcc species, *Burkholderia cenocepacia*, especially the ET12 epidemic strain, is more commonly associated with cepacia syndrome, a rapidly progressing and usually fatal necrotizing pneumonia (3, 4). Most clinically relevant Bcc strains have demonstrated broad-spectrum antibiotic resistance *in vitro* (5). Even with a combination of antibiotics, clearance of infection is not generally observed (6). There is a substantial need to develop new strategies for antimicrobial therapy against this group of pathogens.

One potential strategy is the use of tailocins, or phage-tail like bacteriocins (7). These large bactericidal structures, first identified as R-type and F-type pyocins produced by *Pseudomonas aeruginosa,* resemble phage tails, with the R-type pyocins corresponding to the contractile tails of myophages such as T4 and the F-type pyocins corresponding to the flexible, non-contractile tails of siphophages such as T1. Similar bactericidal complexes were identified in many other bacterial genera (8-10). Recently, the term tailocin has been coined both to highlight the similarity to phage tails and to avoid confusion with the small molecule bacteriocins (11). By extension, tailocins with contractile-tail or flexible tail morphologies are designated as myotailocins and siphotailocins, respectively. Both types of tailocins reproduce the initial steps of a phage infection cycle. After specific adsorption to a phage receptor, there is a conformational change in the tail structure, resulting in the puncturing of the host cytoplasmic

membrane, massive ion release and collapse of the proton motive force. This conformational change is most dramatic in the myotailocin, with the tail sheath contracting, forcing the tail tube through the outer membrane of the host and leading to a puncturing of the inner membrane. In phage infections, ejection of the viral DNA into the cell is followed by resealing of the inner membrane lesion, thus allowing resumption of macromolecular synthesis and the progression of the infection cycle. By contrast, tailocins do not inject DNA, there is no-resealing event, and the cells never recover from the puncturing event, resulting in single-hit, non-lytic lethality.

Studies in the early 1970s showed that *Pseudomonas* myotailocins could be effective as antimicrobial agents of peritonitis in mice (12, 13). However, R-type pyocins exhibit narrow bactericidal spectra (14) and the diversity of tail fibers observed in other systems has shown that tailocins in general have a narrow host range (10). Myotailocins were recently engineered (15, 16) to extend their host range by replacing the C-terminal domain of the tailocin tail fiber (11, 14).

Despite the extensive literatures on bacteriocins, little has been reported about *Burkholderia* bacteriocins and tailocins. Gonzalez and Vidaver (17) first reported bacteriocin production from a small set of *B. cepacia* strains. In a larger study that included 373 isolates from plants, clinical or environmental sources, Govan and Harris (18) developed a typing system based on susceptibility and production of bacteriocins for *B. cepacia* and identified two myotailocins by electron microscopy. More recently, a report addressing the ecological interaction of *P. aeruginosa* and members of the Bcc, which often co-infect patients with CF described that the majority of *P. aeruginosa* inter-specific inhibitory activity was due to the production of myotailocins (19). We report here on the isolation and characterization of a broad host range tailocin from a strain of *B. cenocepacia* and the identification of its cell surface receptors.

## MATERIALS AND METHODS

**Bacterial isolates and culture conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. The panel of 20 *Burkholderia* isolates included 18 *B. cenocepacia* isolates (seven members of the ET-12 lineage, three of the Mid-West clone, eight of the PHDC clone (10)) and one each of *B. cepacia* and *Ralstonia pickettii*, were listed in Table S1. The seventy-three Bcc isolates and the three *B. gladioli* isolates used in sensitivity testing were stock cultures from the U.S. *Burkholderia cepacia* Research Laboratory and Repository (Ann Arbor, MI; Table S2). *Burkholderia glumae* isolates used in the study were obtained from rice field samples, in Texas, expressing bacterial panicle blight symptoms (Gonzalez, unpublished; Table S2). Tryptone nutrient broth (TNB) (20, 21) was used for liquid culture. Tryptone nutrient agar (TNA) used for routine maintenance of cultures was identical to TNB except it lacked $KNO_3$ and was supplemented with 20 g $l^{-1}$ agar. TNA soft agar (TNSA) (TNA with 7.5 g $l^{-1}$ agar) was used for overlays. Luria-Bertani (LB) medium was used in mating experiments (22). For counter-selection, co-integrants were grown in Yeast-Tryptone (YT) broth, which consisted of 10 g $l^{-1}$ of tryptone and 10 g $l^{-1}$ of yeast extract in deionized water. Sucrose was added to YT broth at a final concentration of 15% w/v and supplemented with 20 g $l^{-1}$ agar (YTA) to resolve co-integrants. All *Burkholderia spp, Escherichia coli,* and *P. aeruginosa* strains were grown at 37°C in both liquid and solid medium unless stated otherwise. Antibiotics were added to the media at the following concentrations: 600 µg $ml^{-1}$ kanamycin (Km) and 20 µg $ml^{-1}$ tetracycline (Tc) for *B. cenocepacia;* 30 µg $ml^{-1}$ Km for *E. coli,* respectively.

**Tailocin induction study.** For a typical small batch UV induction**,** strain BC0425 was grown on a TNA plate overnight at 37ºC. A suspension was made and used to inoculate 20 ml TNB ($A_{600}$

= 0.08) and grown with shaking (180 rpm) at 37ºC to $A_{600}$ = 0.5. The cells were pelleted by centrifugation (10,000 X ***g*** for15 min at 5°C), washed once with 0.85% w/v NaCl, repelleted and resuspended in 10 ml of 0.85% w/v NaCl. The suspension was poured into a sterile 100 mm X15 mm Petri dish and with lid off exposed to UV for 7 sec (360 µw $cm^{-2}$ $sec^{-1}$) in a Class II biosafety hood. Ten ml of 2XTNB and 30 ml of TNB were added to the treated and untreated control cells to make the final volume 50 ml. The cultures were returned to 37°C with shaking at 150 rpm. Growth of the culture was monitored using a Nephelo culture flask. When $A_{600}$ decreased below 0.2 for the UV-treated culture, DNaseI was added to a final concentration of 2 U $ml^{-1}$ and the cultures were shaken at 37°C for an additional 30 min. Cell debris was removed by centrifugation (17,418 X ***g*** for 30 min at 5ºC) followed by filtration through a 0.22 µm filter (Millipore, Billerica, MA). Production of pyocins R1, R2 and R5 for spot sensitivity testing was accomplished by UV induction in the same way as described above. Lysates were titered as described below using strain PC184 or PAO1*wbpM* as indicators for tailocin BceTMilo and R pyocins, respectively. To calculate the burst size, the CFU was determined prior to UV exposure and the number of tailocin particles produced was estimated based on Poisson-distribution killing assay, as described below. The tailocin burst size was calculated as total killing units (KU) divided by number of cells at the time of UV induction.

**Tailocin activity assay.** The antimicrobial activity of the tailocin was determined semi-quantitatively by a spot dilution method. Briefly, serial dilutions (twofold, fivefold or tenfold) of tailocin preparations were prepared in P-buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5) and 10 µl of each dilution was spotted on TNA plates that had been overlaid with TNSA

seeded with approximately $10^7$ CFU $ml^{-1}$ of an indicator bacterium. After incubation for 18 h at 37ºC the plates were evaluated for zones of inhibition. The reciprocal of the highest dilution that formed a visible inhibition zone was defined as the relative activity in arbitrary units (AU).

Quantitative tailocin activity assays were performed using a Poisson-distribution killing method, adapted from Williams *et al.* (14), originally described by Kageyama *et al.* (23). Briefly, tailocin serial dilutions in P-buffer were added to $10^9$ CFU $ml^{-1}$ target bacteria in TNB and incubated for 40 min at 37°C in triplicate. The samples were then serially diluted and plated on TNA to count surviving bacteria. The number of tailocin particles is related to the fraction of bacterial survivors in a Poisson distribution, $m = -\ln(S)$, where $m$ is the possible average number of lethal event (i.e., adsorbed tailocin particles) per bacterial cell, and $S$ is the fraction of survivors. The total number of active particles $ml^{-1} = m$ x cells $ml^{-1}$ was defined as the KU $ml^{-1}$, and is based on the assumption that in each sample, adsorption was quantitative within the 40 min incubation period.

**Large scale tailocin production and purification.** The method of Scholl *et al*. (7) was used for large-scale preparation. Briefly, ammonium sulfate was slowly added to the filter-sterilized tailocin lysate to a final concentration of 40% (w/v) with stirring on ice. After 18 h at 5ºC, the ammonium sulfate precipitate was pelleted by centrifugation at 17,418 X ***g*** for 1 h at 5ºC and the pellet was resuspended in 1/10 of the original volume in cold $TN_{50}$ (10 mM Tris, pH7.5, 50 mM NaCl) buffer. The suspension was dialyzed overnight against $TN_{50}$ buffer using a Slide-A-Lyzer® dialysis cassette (3.5 kDa MWCO; Pierce). The tailocin particles were sedimented at

90,619 X ***g*** for 2.5 h at 5ºC using a Beckman type 60Ti rotor; the pellet was resuspended in P-buffer and titered for activity as described above.

**Preparative Isoelectric Focusing.** For purposes of imaging and protein analysis, further purification was performed. Preparative isoelectric focusing (IEF) generated acceptably pure tailocin preparations. For IEF, 200 ml of an UV induced tailocin lysate was concentrated by ultracentrifugation (90,619 X ***g*** for 2.5 h at 5ºC) using a type 60Ti rotor in a Beckman L8-70 M ultracentrifuge. The pellet was resuspended in 2.5 ml sterile ultrapure water (Invitrogen) containing 0.3% (w/v) octylglucoside and 5% (v/v) glycerol. Three ml of Bio-Rad Ampholyte (3-10) was added to a buffer containing 3M Urea, 0.3% octylglucoside, and 0.3% (w/v) CHAPS **(**3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) to a final volume of 50 ml**.** Anion exchange membranes were equilibrated in 0.1 M NaOH and cation exchange membranes in 0.1 M $H_3PO4$. The tailocin preparation was applied to a Rotofor chamber (BioRad) that had been pre-focused at 15 W constant power for 1 h. The chamber was run at 15 W at 4ºC until the current stabilized. The pH of each fraction (~20 fractions) was determined. The fractions were adjusted using 1 M Tris-HCl pH 7.0 to a final concentration of 0.1 M, and the activity was measured by spot-dilution method as described above.

**Electron microscopy.** IEF purified tailocin sample (2 X $10^{11}$ KU $ml^{-1}$) diluted 1:5 in P-buffer was negatively stained for transmission electron microscopy (TEM) as described by Ahern *et al*. (21). Specimens were observed on a JEOL 1200EX TEM (Microscopy and Imaging Center, Texas A&M University) operating at an acceleration voltage of 100 kV. Images were recorded at

calibrated magnifications by a charge-coupled device (CCD) camera, and measurements were acquired using Image J software.

**Effects of heat and enzyme treatments.** The temperature stability of tailocin BceTMilo was assessed by heating a large-scale purified tailocin preparation ($10^{10}$ KU $ml^{-1}$) to 50ºC and 80ºC for 60 min, or 100ºC for 3 and 10 min. Samples were immediately chilled on ice after heating and assayed for activity by the spot-dilution assay as described above.

Enzymatic stability of the tailocin to trypsin (131,000 U $mg^{-1}$), α-chymotrypsin (83.9 U $mg^{-1}$), proteinase K (34 U $mg^{-1}$), protease (4 U $mg^{-1}$), lipase (20,000 U $mg^{-1}$), papain (24 U $mg^{-1}$), or lysozyme (70,000 U $mg^{-1}$) were assayed using conditions previously described by Gonzalez & Kunka (Gonzalez & Kunka, 1987). All enzymes were purchased from Sigma. Tailocin preparations ($10^{10}$ KU $ml^{-1}$) were incubated with each enzyme at a final concentration of 500µg $ml^{-1}$. Enzyme treatments with α-chymotrypsin and trypsin were performed at 25ºC for 60 min, with all other enzyme/tailocin mixtures incubated at 37ºC for 60 min. Tailocin activity, after incubation with individual enzymes, was assayed as described above.

**pH stability.** Tailocin prepared using the large-scale method was dialyzed against buffers ranging from pH 2-10 using a Slide-A-Lyzer® dialysis cassette (3.5 kDa MWCO). The tailocin solution was dialyzed for 18 h with two exchanges against each of the following buffers: 0.05 M glycine hydrochloride buffer (pH 2.04), 0.05 M citrate buffer (pH 4.78), 0.05 M Tris-HCl buffer (pH 6.8 and 8.8), and 0.05 M carbonate-bicarbonate buffer (pH 10.64). The control was dialyzed against P-buffer. The contents were then serially diluted with P-buffer and assayed for activity

as described above. All buffers were spotted to overlays to confirm killing activity was not due to buffer activity.

**SDS-PAGE.** The major proteins of tailocin BceTMilo were separated using SDS-PAGE gel electrophoresis. Eight µg of IEF purified tailocin sample were added to 20 µl of SDS loading buffer [50 mM Tris-HCl (pH 7.5) with 0.01 g of SDS, 1.54 mg of dithiothreitol, and 0.1 mg $ml^{-1}$ of bromophenol blue], heated at 95°C for 5 min, and applied to either a 7.5% or a 10-20% gradient Tris-HCl Mini-PROTEAN gel (BioRad, Hercules, CA). Electrophoresis was carried out at 200 V (constant) as described by Laemmli (24). Precision Plus protein standard (Bio-Rad/161-0374) was used as a standard. The gels were stained with Coomassie Brilliant Blue R250 (BioRad).

**Liquid Chromatography-tandom Mass Spectrometry (LC-MS/MS) analysis**. To conduct LC-MS/MS analysis of the tailocin major proteins, the 43 kDa and 17 kDa protein bands were excised from a 10-20 % Tris-HCl gel, and the 100 kDa and 30 kDa bands were excised from a 7.5% SDS Tris-HCl gel. The proteins were subjected to LC-MS/MS analysis on a Q-TOF Premier Mass Spectrometer (University of Michigan Biomedical Core Facility- Proteomics and Peptide Synthesis Core). Protein LynX *g*lobal Server and Mascot search engines were used to search the SwissProt (http://ca.expasy.org/sprot) and NCBI databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (25, 26).

**DNA manipulations.** Genomic DNA was extracted using a DNeasy kit (Qiagen, Valencia, CA), plasmid DNA was prepared using a Miniprep kit (Qiagen), gel extractions and PCR product purification were conducted using a Qiaquick gel extraction kit and Qiaquick PCR purification kit, respectively, according to manufacturer's instructions (Qiagen). All restriction endonucleases, *Taq* DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA) and were used according to manufacturer instructions. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). DNA sequencing was performed at the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University.

**Sequencing assembly and annotation of BceTMilo genes.** To understand the genetic organization of BceTMilo, BC0425 genomic DNA was isolated using modifications of the CTAB method (27). BC0425 DNA was sequenced using 454 genome sequencing technology (Duke University, Institute for Genome Sciences & Policy). Sequence information was assembled into contigs using the Newbler sequence assembly program (Roche, USA). Genemark http://exon.biology.gatech.edu/gmhmm2_prok.cgi) was used to predict all possible open reading frames. To annotate contig 77, which contains the BceTMilo genes, Genemark (http://exon.gatech.edu/GeneMark/heuristic_gmhmmp.cgi) was first used to identify all possible ORFs (28). The Artemis Comparison Tool (Sanger Centre) was then used to identify the most likely start codon for each ORF, by searching for the presence of a potential ribosomal binding site (RBS) (29). Each identified ORF was then compared to the NCBI protein database using Blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to assign putative functions (25). InterProScan

(http://www.ebi.ac.uk/Tools/webservices/services/interproscan), LipoP (http://www.cbs.dtu.dk/services/LipoP/), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) were used to identify conserved domains, lipoprotein processing signals, and transmembrane domains (TMDs), respectively. Genome maps were drawn utilizing the programs DNA Master (http://cobamide2.bio.pitt.edu/computer.htm) and Inkscape (https://inkscape.org/en/).

**Lipopolysaccharide extraction.** Lipopolysaccharides (LPS) extracts from tailocin sensitive and resistant strains were obtained using the hot phenol method as described by Apicella *et al*. (30). The dry weight of purified LPS was determined and resuspended in endotoxin free water (Sigma) to a concentration of 1-2 µg/ml.

**Electrophoretic analysis of LPS.** LPS samples were analyzed using the Laemmli SDS-gel electrophoresis system (24). LPS preparations were dissociated in SDS loading buffer and heated at 90-100ºC for 5 min. LPS was resolved by running 1.0 µg of each extract on a 16.5% Tris-Tricine SDS mini-gel (Bio-Rad) for 6 h at 60 volt and visualized by silver staining (31).

**LPS adsorption assay.** A LPS adsorption assay was performed using a purified tailocin preparation ($10^{11}$ KU $ml^{-1}$). The tailocin preparation was diluted (1:10) with P-buffer amended with 5mM $CaCl_2$ (PC-Buffer) and mixed with increasing amounts of LPS preparations. After incubation at 37ºC for 30 min, the mixtures were assayed for activity by spotting a 1:2 dilution series on overlays seeded with *B. cenocepacia* strain PC184, as described above.

**Sugar inhibition assay.** The sugar inhibition assay was performed as described by Dawes (32) with minor modifications. The effects of D-galactose, α-D-glucose, D-fructose, D-Mannoheptose, L-rhamnose, D-cellobiose, lactose, sucrose, and D-raffinose on the adsorption of tailocin BceTMilo to strain PC184 were tested. Strain PC184 was grown in 1 L TN broth (200 rpm) to an $A_{600}$ = 1. Cells were centrifuged (10,000 X ***g***) at 4°C for 5 min. The cells were washed in 40 ml P-buffer, centrifuged, and then resuspended in 4 ml P-buffer. Two ml of the bacterial suspension ($10^{11}$ cells $ml^{-1}$) was mixed with 500 µl of tailocin BceTMilo ($10^{9}$ KU $ml^{-1}$) and 2.5 ml of 1.2 M sugar (final concentration of 0.6 M). The mixture was incubated at 37°C and 500 µl samples were taken at 0, 1, 3, 6, and 9 min post mixing. Samples were immediately centrifuged and supernatants were filter sterilized. The remaining tailocin activity of the samples was determined using a spot assay. The percentage of unadsorbed tailocins was calculated by dividing the titer of each sample by the titer of the buffer control.

**Construction of deletion mutants in *B. cenocepacia* BC0425 and K56-2.** Deletion of *recA* in the BceTMilo producer strain BC0425, and the *waaL* and *waaC* in indicator strain K56-2 were performed using the system developed by Hamad *et al*. (33), except that the constructed suicide vectors (pMo130::*recA*-UD, pMo130::*waaL*-UD and pMo130::*waaC*-UD ) were introduced into *B. cenocepacia* strain BC0425 or K56-2 by conjugation using tri-parental mating with pRK2013 as the mobilizing plasmid. In brief, donor, mobilizer, and recipient suspensions were made in LB broth from cultures grown on solid media under selective conditions, as appropriate for 18 h. Bacterial suspensions were adjusted spectrophotometrically to $A_{600}$= 0.5, mixed at an equal ratio (1:1:1), and transferred to a positively charged sterile membrane layered on a 100 x 15 mm LB

agar petri dish. Following an 18 h incubation period at 37°C, the cells from the mating and respective controls were washed twice in phosphate buffer (0.125 M, pH 7.1) by centrifugation (12,096 X *g* for 10 min at 5°C). The bacterial pellets were resuspended in phosphate buffer and dilutions were plated on LB agar plates amended with 600 µg $ml^{-1}$ Km and 20 µg $ml^{-1}$ Tc for selection of single crossover events. PCR primers for regions flanking *recA* were designed based on the annotated sequence of BC0425. Since *B. cenocepacia* strain K56-2 is clonally related to the sequenced strain J2315 (34, 35), PCR primers for regions flanking *waaL* and *waaC* in strain K56-2 were designed based on J2315 sequences BCAL2404 to BCAL2406 and BCAL3111 to BCAL3113, respectively (www.sanger.ac.uk/Projects/B_cenocepacia/). Primers used for constructing the suicide vectors (pMo130::*recA*-UD, pMo130::*waaL*-UD and pMo130::*waaC*-UD) were listed in Table 2.. The deletions of the respective genes were confirmed by PCR.

**In-*trans* complementation of BC0425 and K56-2 mutants.** Plasmids pMo168::*recA*-comp, pMo168::*waaL*-comp and pMo168::*waaC*-comp were constructed as replicative vectors for in-*trans* complementation of BC0425 Δ *recA*, K56-2 Δ *waaL* and K56-2 Δ *waaC* mutants, respectively. Plasmids were introduced into the deletion mutants by tri-parental mating as described above. Primers used for amplifying the wild-type *recA*, *waaL* and *waaC* were listed in Table 2. The presence of the wild-type *recA*, *waaL* and *waaC* were confirmed by PCR.

**Nucleotide sequence accession number.** In accordance with Texas A&M Center for Phage Technology policy, the tailocin from *B. cenocepacia* BC0425 was designated BceTMilo **(**https://cpt.tamu.edu**).** The genome was deposited in GenBank under the following accession number: KY316063.

## RESULTS

**Identification, induction and morphology of tailocin BceTMilo.** In the process of screening for tailocins active against members of the Bcc, it was observed that *B. cenocepacia* BC0425 produced small clear zones of inhibition on overlays of 11/20 panel indicator isolates (Table S1). Cultures of BC0425 induced with UV light underwent lysis and produced ~5 X $10^{10}$ KU/ml$^{-1}$, indicating with the presence of an inducible tailocin locus (36, 37, 38). Tailocins could be purified and concentrated by ammonium sulfate precipitation followed by ultra-centrifugation, achieving yields of ~40% and final titers of ~5 X $10^{11}$ KU ml$^{-1}$.

The tailocins further purified by preparative isoelectric focusing and visualized by transmission electron microscopy, displayed the characteristic morphology of R-type pyocins of *P. aeruginosa* (37). Electron micrographs of purified tailocin showed both uncontracted (~87.4%) and contracted (~12.6%) particles (Fig. 1A). The uncontracted particles were 141 ± 3.5 nm by 17± 1 nm rods, with a baseplate and several tail fibers visible in the electron micrograph (Fig. 1B, arrows), and ~33-34 rings of sheath proteins, as estimated by direct counting (not shown). Some particles were contracted, resulting in a shortening and thickening of the sheath (55 nm x 23 nm) and the exposure of a 140 nm x 8 nm tail tube (Fig. 1A). Based on the morphology, the myotailocin was designated as BceTMilo, where “Bce” indicates the host (*B. cepacia*), “T” designates tailocin (rather than phage), and Milo is the assigned name to the locus (based on our

convention that the names for phage and phage-derived particles with contractile, flexible or short/stubby tails begin with M (myo), S (sipho) and P (podo), respectively (https://cpt.tamu.edu).

**Characterization of the myotailocin BceTMilo.** Purified BceTMilo did not lose killing activity when exposed to trypsin, α-chymotrypsin, proteinase K, protease, lipase, papain, or lysozyme, consistent with other tailocins (38). BceTMilo killing activity was stable for 1 h at 50ºC, but was lost after 1h at 80ºC, or 3 min at 100ºC; when stored at 4-8 $^{0}$C, the killing activity had ~ 50% loss over a six-month period. Tailocin killing activity was stable between pH 4.8 - 8.8, with a 100-fold decrease in activity after 18 h at pH 10.5 and all detectable activity lost after 18 h at pH 2.0.

SDS-PAGE analysis of IEF-purified tailocin revealed four major protein subunits of 17, 30, 43 and 100 kDa (Fig. 2A&2B). For myotailocins, which have similar structures to the tails of myophages like T4, the two most numerous species should be the sheath and tube proteins, which typically have ~40 kDa and ~20 kDa molecular masses (39) corresponding to the 43 kDa and 17 kDa species (Fig. 2A). Given the 33-34 rings of sheath proteins as determined above, both sheath and tube proteins should be present at ~200 molecules per tailocin particle. Densitometry analysis of the 43 kDa and 17 kDa tailocin protein species allowed quantification of tailocin particles and a calculation of their killing efficiency (Table 3). Based on this, given the limitations of Coomassie Blue staining, we estimated an average efficiency of killing (EOK) of ~0.5 (Table 3). This strongly indicates that BecTMilo kills bacterial targets by the same single-hit killing mechanism characterized for other myotailocins (7). Based on this estimate, it is possible to approximate that induced cells on average produce 620 +/- 30 KU or particles cell$^{-1}$ (Table 4).

**Mapping and features of the BceTMilo defective prophage.** To determine the genetic locus encoding the tailocin, a draft genome of strain BC0425 was compared to peptide sequences obtained from LC-MS/MS analysis of the tail sheath and tube proteins, as well as from the two minor proteins. All four proteins could be mapped to a 23 kb contig of the draft genome (Fig. 3A), which contained 30 predicted phage-related genes (Table S3). The same element was found in the fully annotated genome of *B. cenocepacia* J2315 (Fig. 3A) and many other Bcc strains (Fig. S1), with the J2315 locus sharing >99% sequence identity over most of the genes, except for a 1.9kb insertion, identified as a group II intron, between genes *8* and *9* (Fig. 3A).

A syntenic arrangement of genes was also found in the Bcc myophage KL3 (40), a phage obtained as a spontaneous plaque-former in a lawn of its own lysogen; KL3 is shown in its prophage form in Fig. 3B. The morphogenesis genes of KL3, and thus also BceTMilo, are mostly syntenic to the well-studied paradigm temperate myophage P2 of *E. coli* (Fig. 3B), making it possible to assign the function of most of the tailocin genes. For example, BceTMilo genes *16* and *17*, which encode the 17KDa and 43KDa major tailocin species, correspond to the tail tube and sheath genes of KL3 and P2. Also, the genes encoding the 100 kDa and 30 kDa minor species of the tailocin are syntenic to the tail fiber and tail fiber chaperone genes of KL3 and occupy the same positions in the late gene transcript pattern as the same but unrelated genes in P2 (Fig. 3B). As expected, the BceTMilo locus lacks the distal end of the KL3 prophage containing the head morphogenesis and DNA modification genes. Remarkably, the breakpoint in synteny between the tailocin locus and the morphogenesis gene region of KL3 and P2 is between the most distal tail protein, the tail completion protein X, and the first capsid protein, the head completion protein L. The only tail morphogenesis gene present in KL3 and P2 but missing

in the tailocin locus is gene *S*, which was apparently deleted along with a moron element (41) encoding a DNA methylase inserted in the antisense orientation within the tail morphogenesis genes. In P2, the S protein appears to fulfill the role of an adaptor required to allow the completed tail to dock with the filled capsid (42) a function not required for a tailocin. Overall, all the BceTMilo proteins involved in tailocin morphogenesis are also similar to the equivalent genes in the *P. aeruginosa* R-type pyocin locus, except for the tape measure and the tail fiber, and the associated chaperones (not shown).

BceTMilo and KL3 also have very similar lysis genes including a type I holin (*gp29*), a putative antiholin with four predicted TMDs (gp28), and the two spanin subunits (gp25 and 26 for the i-spanin and o-spanin subunit, respectively), as described by Lynch et al (40). These lysis genes are unrelated to the P2 functional equivalents.

Combining synteny and sequence similarity, every BceTMilo gene could be assigned a counterpart in KL3, except for genes *1* and *2*. *gp1* encodes a tyrosine integrase with strong similarity to the P2 Int, but was not detectably related to gp*1* of KL3, also an integrase of the same family. The overlap of gene *2* with the integrase gene and the fact that its product has winged-helix DNA binding motifs suggest that *gp2* encodes a Xis protein required for prophage excision. The only gene that cannot be robustly assigned a function is gene *3*, which has a homolog in a similar *Burkholderia* phage BEK (Database: GenBank Entry: CP008753). The corresponding ORF in KL3 was not previously annotated and was assigned gene *2a* in Fig. 3B.

BceTMilo shares its late gene regulation system with KL3 and P2, since all three encode the well-characterized Ogr transcription factor (43). The genes involved in lysogenic regulation of the KL3 prophage have not been precisely defined, but *gp 9* of the BceTMilo tailocin locus was

393 closely related to *gp13* of KL3 and encodes a CI-like repressor. Induction of BceTMilo was
394 determined to be RecA dependent, as UV induction of the BC0425 Δ *recA* resulted in no
395 production of the tailocin or lysis of cells in parallel experiments where the *in- trans* complement
396 [ BC0425 Δ *recA* (pMo168:: *recA*-comp)] produced near parental levels of tailocin as determined
397 by spot-titer method (data not shown).
398 Comparison of BceTMilo with the tailocin locus in J2315 showed that in addition to the 1.9
399 kb group II intron insertion between the two regulatory genes for J2315 tailocin, there was a 36
400 bp insertion in the J2315 version of the tail fiber assembly gene, resulting in the expansion of
401 Ser-Glu-Pro motif repeats already present in BceTMilo; this motif expansion lies in a position
402 expected to influence host range. Moreover, there were a total of 121 single nucleotide
403 polymorphisms (SNPs) over the 23 kb sequence. Most of the SNPs were shown in gaps between
404 genes. All of the SNPs in genes preserved the reading frame; i.e., all were either silent or were
405 missense changes. Out of 30 missense SNPs, only 3 were detected in hypothetical genes of
406 unknown function; others were in structural genes or in lysis cassettes. For example, there were 7
407 SNPs in the tail fiber gene and 8 SNPs in the tape measure protein detected, which represented
408 5.2 kb of the locus; and 2 and 3 SNPs were found in i-spanin/o-spanin and endolysin genes,
409 respectively. Tailocin activity has not been reported for J2315, which contains the fully
410 functional BcepMu prophage (44). However, negative-stain electron microscopy of concentrated
411 lysates obtained from UV-induction of J2315 revealed low numbers of tail-like structures
412 indistinguishable from the BceTMilo particles for which we were unable to find a sensitive host
413 among 41 Bcc strains tested. Nevertheless, the stringent conservation of all the structural genes

suggests that the tailocin encoded in J2315 may contribute to fitness, and thus may be functional but with a different host range dictated by the repeat expansion in the tail fiber assembly gene.

**Inhibitory spectrum of tailocin BceTMilo.** To determine the inhibitory spectrum of the tailocin, a panel of 101 *Burkholderia* species was tested for sensitivity, using both broth micro-dilution and spot assays. Of the 73 isolates representative of the species belonging to the Bcc, 50 were sensitive to BceTMilo (Table S2), with 59% of *B. cenocepacia* isolates tested exhibiting sensitivity. The non-Bcc *Burkholderia* species assayed, *B. gladioli* and *B. glumae,* showed high sensitivity (93%; Table S2). Overall 76% of *Burkholderia* species tested were sensitive to tailocin BceTMilo. A subset of the same *Burkholderia* isolates panel were spot tested for the susceptibility to pyocins R1, R2 or R5, only 0/47, 1/47 and 4/47 were sensitive to each of the respective pyocins (Table S2).

**LPS determinants as receptors for BceTMilo.** To determine the nature of the receptor for tailocin BceTMilo, LPS adsorption assays were conducted. LPS from a sensitive strain (PC184) and a resistant strain (AU10487) was extracted and used for the assay. Tailocin activity decreased in mixtures with increasing concentrations of LPS from the sensitive strain (Fig. 4A), whereas no decrease in activity was observed in mixtures with increasing concentration of LPS from the resistant strain (Fig. 4B), indicating that the receptor for BceTMilo was part of the LPS of the sensitive strain.

Since a sugar moiety(s) of LPS could be involved in adsorption of tailocin BceTMilo, a sugar inhibition assay was conducted. Sugars α-D-glucose, L-rhamnose, and D-manno-heptose were

436 chosen for evaluation since they are components of *B. cenocepacia* LPS (45, 46). Sugars α-D-
437 glucose and α-L-rhamnose inhibited tailocin adsorption, whereas D-galactose and D-fructose had
438 little or no effect on tailocin adsorption to sensitive cells (Fig. 5). The effects of di- and tri-
439 saccharides containing glucose were also investigated. Sucrose and raffinose, both with a α-1, 2
440 glycosidic linkage, inhibited adsorption, whereas D-cellobiose and lactose, both with a β-1, 4
441 glycosidic linkage, showed only minor inhibition (Fig. 5).

442 Kohler *et al.* (47) have proposed that LPS plays an essential role both as a protective shield
443 and as a receptor for R-pyocins of *P. aeruginosa*. Using genetically defined LPS mutants, they
444 showed that the L-rhamnose residue was the receptor for R1 in *P. aeruginosa* and that two
445 distinct D-glucose residues of the outer core were part of the receptor sites for R2- and R5-
446 pyocins. We demonstrate here that *P. aeruginosa* strain PAO1 exhibited slight sensitivity to
447 tailocin BceTMilo (Fig. 6A (i), 6B (i), zones not clearly visible), whereas the PAO1 *wbpM*
448 mutant derivative, deficient in B-band, showed increased sensitivity to tailocin BceTMilo (Fig.
449 6A (ii), 6B (ii)). The *wbpL* derivative of PAO1, deficient in both LPS B-band and A-band, and a
450 PAO1 *rmlC* devoid of L-rhamnose, but containing an α-glucose residue, were both sensitive to
451 BceTMilo (Fig. 6A (iii), 6B (iii), 6A (iv), 6B (iv)). However, an *algC* mutant, devoid of both an
452 L-rhamnose and α-D-glucose residues exhibited no sensitivity to BceTMilo (Fig. 6A (v), 6B (v)),
453 indicating that α-D-glucose is required for adsorption of tailocin BceTMilo in *P. aeruginaosa*.

454 LPS mutants of the genetically malleable *B. cenocepacia* strain K56-2 and their complements
455 were constructed in this study and their susceptibilities to BceTMilo were tested. LPS extracted
456 from K56-2, J2315 or K56-2 deletion mutants, and their complements were compared using SDS
457 gel electrophoresis. Deletion of the *waaL*, an O-antigen ligase, predicted a loss of the O-antigen

458 surface expression and the L-rhamnose in K56-2 (46), and deletion of *waaC*, a
459 heptosyltransferase I, would result in a heptose-less mutant with a rough phenotype. A ladder-
460 like banding pattern, corresponding to the presence of polymeric O-antigen, was visible for strain
461 K56-2, whereas J2315 was devoid of an O-antigen (Fig. 7, lanes 1 and 2, respectively) as was
462 previously reported by Ortega (48). The absence of O-antigen ladder was observed in the K56-2
463 Δ *waaL* preparation (Fig. 7, lane 3) and a severely truncated lipid-A-core region with the
464 concomitant loss of O-antigen production was observed in the K56-2 Δ *waaC* mutant (Fig. 7,
465 lane 4). The in-*trans* complementation of K56-2 Δ *waaL* restored O-antigen production (Fig. 7,
466 lane 5) and in-*trans* complementation of K56-2 Δ *waaC* restored O-antigen production and lipid-
467 A core regions (Fig. 7, lane 6). Using the spot assay, we demonstrated that strains K56-2, J2315,
468 and the K56-2 *waaL* deletion mutant (Δ *waaL*) were sensitive to tailocin BceTMilo (Fig. 8A (i),
469 8A (ii) and 8A (iii)), but the Δ *waaC* mutant of K56-2 (Fig. 8A (iv), 8B (iv)). The
470 complementation of K56-2 Δ *waaL* deletion did not change the sensitivity to BceTMilo (Fig. 8A
471 (v)), but the complementation of K56-2 Δ *waaC* deletion restored the sensitivity to BceTMilo
472 (Fig. 8A (vi)).
473

474 **Discussion**

475 The Bcc and *P. aeruginosa* are significant opportunistic human pathogens in CF patients (35).
476 Early lung infections in CF patients are most often due to *P. aeruginosa;* however, secondary
477 infections caused by Bcc often give rise to a highly variable and unpredictable clinical outcome
478 (49). Previous studies have suggested that *P. aeruginosa* and Bcc actively affect each other's
479 growth through production of secreted products (50). Several studies have been reported that
480 most clinical and environmental *P. aeruginosa* strains produce pyocins (19, 51, 52), whereas
481 there is only limited information concerning bacteriocins produced by *Burkholderia* species (16,
482 17, 19). Bakkal *et al*. (4) noted that almost all *P. aeruginosa* isolates in their study were able to
483 inhibit a wide range of *P. aeruginosa* and Bcc strains, whereas Bcc strains, on average, inhibited
484 a much more limited number of strains.

485 In this study, we identified and characterized the tailocin BceTMilo, which exhibited broad
486 host activity among *Burkholderia* spp. Evaluation of BceTMilo showed that 68% of Bcc
487 representing 10 species and 90% of non-Bcc *Burkholderia* strains tested were sensitive (Table
488 S2). Additionally, tailocin BceTMilo had killing activity against *P. aeruginosa* PAO1 and
489 derivatives.

490 The observed average of 600 particles per cell of BceTMilo produced by *B. cenocepacia*
491 strain BC0425 is three to six fold higher than the 100-200 particles/ cell reported for production
492 of pyocin R2 by strain PAO1(7). In *P. aeruginosa* the RecA, PrtR (repressor protein) and PrtN
493 (activator protein) co-regulate expression of R-, F-, and S-type pyocin genes that are induced in
494 responses to such mutagenic agents as UV and mitomycin C (54) or oxidative stress (55).
495 Genome-wide transcriptome analysis of *P. aeruginosa* has revealed that pyocins S-, F-, and R

transcripts are upregulated by oxidative stress as are lysis related genes, indicating that multiple pyocins are released upon induction (55). This coordinated transcription, translation and lysis of cells may limit the number of R-type pyocin particles produced per cell.

The synteny and close relationship between BceTMilo, KL3 and, by extension, the paradigm temperate myophage P2 suggest that, other than regulatory genes, nothing more is needed to generate a tailocin other than the deletion of the head genes (Fig. 3B). Since every structural gene required for the P2 myophage tail has a homolog both in KL3 and in the tailocin locus, the simplest explanation could be that both BceTMilo myotailocin locus and the temperate myophage phage KL3 were derived from a common ancestor, with BceTMilo undergoing internal deletions of the head and DNA modification genes.

Comparison of BceTMilo with a tailocin locus in the epidemic strain J2315 showed that more than 99% sequence identity in genes, with the exceptions of three areas. There was a 1.9 kb group II intron insertion between the two regulatory genes for J2315 tailocin, a 36 bp insertion in the J2315 version of the tail fiber assembly gene, and there were a total of 121 SNPs over the 23 kb sequence. The group II intron insertion between the two regulatory genes may have affected transcription efficiency and that resulted in the observed low production of particles from J2315. The observed SNPs were dispersed throughout the J2315 tailocin cassette and did not result in frameshifts that would affect protein expressions. As might be expected, the largest observed difference between the BceTMilo and J2315 tailocin was in the tail fiber assembly gene, which determines the specificity of the two tailocins and therefore their host range spectrums.

A critical feature for pyocins/tailocins is their ability to recognize and bind cell receptors specific to their host. Different bacterial surface components act as the receptors for phages and

phage-tail like bacteriocins, including flagella (56), pili (57), outer membrane proteins such as OmpA and OmpC (58) and LPS (47, 59). LPS adsorprion assay showed that the activity of tailocin BceTMilo was neutralized by purified LPS from sensitive strains, but not from resistant strains (Fig. 4). Sugar inhibition assays indicated that L-rhamnose and sugars in which D-glucose was α-linked to another residue inhibited adsorption (e.g. sucrose or raffinose), whereas sugars with a β-linked D-glucose, as in D-cellobiose or lactose, did not inhibit adsorption (Fig. 5). This suggested that both α-D-glucose and L-rhamnose were the putative sugar components of the LPS involved in BceTMilo adsorption.

Our evaluation of BceTMilo for activity against PAO1 and LPS mutant derivatives showed that the parental strain, expressing both the A-band and B-band O polysaccharides were less sensitive than PAO1*wbpM* (LPS $A^+$ LPS $B^-$) and PAO1*wbpL* (LPS $A^-$ LPS $B^-$), indicating that removal of the B-band LPS exposed the receptor site for the BceTMilo. The PAO1 *rmlC* mutant lacking the LPS A and B bands and a terminal L-rhamnose, but containing a α-D-glucose residue was sensitive to BceTMilo, whereas the PAO1*algC* mutant, devoid of both bands as well as the terminal L-rhamnose and α-D-glucose residues was resistant, suggesting that α-D-glucose is a receptor for adsorption of tailocin BceTMilo in *P. aeruginosa*.

The results obtained with PAO1 were correlated with the sensitivity patterns observed in *B. cenocepacia* J2315, K56-2 and its derivative LPS mutants (Fig. 8). J2315 contains a terminal α-D-glucose and L- rhamnose and showed sensitivity to BceTMilo, as did K56-2 that contains an internal α-D-glucose in the core oligosaccharide and L- rhamnose as part of the O-antigen. The K56-2 Δ *waaL* mutant (O-antigen deficient) devoid of L- rhamnose exhibited sensitivity to BceTMilo, which argues against the role of L- rhamnose as a receptor, despite the sugar

inhibition data. Furthermore, the K56-2 Δ *waaC* mutant (O-antigen and α-D-glucose deficient) lacking the internal α-D-glucose was resistant to BceTMilo, which strongly suggests that α-D-glucose either internal or terminal position is the receptor for the tailocin in K56-2 or J2315.

The host range activity of tailocins is determined by their tail fibers and generally results in a narrow spectrum of killing activity (14, 16). Tailocin BceTMilo exhibited broad host activity among *Burkholderia* species and lineages (Table S2). Bakkal *et al.* (19) noted that almost all *P. aeruginosa* isolates in their study were able to inhibit a wide range of *P. aeruginosa* and Bcc strains, whereas Bcc strains, on average, inhibited a much more limited number of strains, suggesting that bacteriocin-based inhibition may play a role in governing *P. aeruginosa* and Bcc interactions in the CF lung. Our testing of 44 Bcc isolates, representing nine species using pyocins R1, R2 or R5 lysates indicate very limited activity by the R-pyocins toward the Bcc (Table S2). Given that R2 encompasses the spectra of R3 and R4 (15, 60), we submit that all interspecific activity observed against the Bcc by Bakkal *et al.* (19) may not have been due to pyocin activity and may be a reflection of the isolates used in the study. Clearly more information is needed to determine the role tailocins play in the dynamics of microbial interactions.

Tailocins have previously been recognized as potential anti-bacterial agents (7, 61). The disadvantage of tailocins is obviously the incapacity for proliferation at the site of therapeutic action, thus requiring delivery at high concentration. However, this disadvantage is somewhat compensated for by two fundamental aspects of tailocin character: the lack of DNA, thus ameliorating concerns related to the unintentional spread of genes through specialized or generalized transduction or the dispersion of recombinant genetic material; and the one-hit

562 killing character, which allows the definition of dose in pharmacological usage. A further,
563 somewhat less obvious limitation has been the rather narrow host range of the well-described
564 tailocins, which may be related to the fact that hosts carrying tailocin loci cannot rely on DNA-
565 based immunity for protection against the homotypic tailocin. Here, we have described a board
566 host-range tailocin with activity against both human and plant pathogenic members of the genus
567 *Burkholderia*. Recent studies with modified R-type tailocins have shown therapeutic promise in
568 a murine model of *P. aeruginosa* peritonitis (7) and in *Clostridium difficile* infection (61).
569 Evaluations of BceTMilo in both an animal and plant models are needed to determine its
570 potential as a potential treatment for *Burkholderia*.
571

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**Figure legends**

**FIG 1** Transmission electron micrographs of IEF purified tailocin BceTMilo. Samples were negatively stained with 2% (wt/vol) aqueous uranyl acetate. Bar indicated 100 nm. (A) X 25 K, contracted tailocin were shown. (B) X 100 K, arrows indicated tail fibers.

**FIG 2** SDS-PAGE of the IEF-purified tailocin BceTMilo. Protein subunits of tailocin BceTMilo were dissociated as described in Materials and Methods and loaded on (A) 10-20% Tris-HCl SDS gel or (B) 7.5% Tris-HCl SDS gel. Lane 1, molecular mass standards (Precision Plus protein standard from Bio-Rad; 10–250 kDa); Lane 2, subunits of tailocin BceTMilo. Proteins were visualized using Coomassie blue staining. Please note that the white lines between lanes 1 and 2 for both Figure 2A and 2B indicate that a lane was removed from each of the original gels, since data was not presented in Results.

**FIG 3** Genomic map of BceTMilo and syntenic comparison. (A) Syntenic relationship of tailocin BceTMilo compared to tailocin locus on *B. cenocepacia* J2315. (B) Syntenic comparison of tailocin BceTMilo, *Burkholderia* phage KL3 and *Enterobacteria* phage P2. The genomic maps were drawn to scale. Genes were color coded according to Dice amino acid similarity compared to *Burkholderia* phage KL3 and the related genes were connected with dotted lines.

**FIG 4** Effect of *Burkholderia* LPS on BceTMilo. Increasing amounts of purified LPS from (A) *B. cenocepacia* strain PC184 (sensitive) and (B) *B. cenocepacia* strain AU10487 (resistant) were mixed with tailocin BceTMilo and allowed to incubate for 30 min. A 1:2 dilution series (See

771 Materials and Methods) of mixtures from (A) and (B) were spotted to overlays seeded with strain
772 PC184 to determine residual BceTMilo activity. Results are representative of triplicate
773 experiments.
774
775 **FIG 5** Sugar inhibition assay. Effects of D-galactose, α-D-glucose, D-fructose, D-
776 Mannoheptose, L-rhamnose, D-cellobiose, lactose, sucrose, and D-raffinose on tailocin
777 adsorption to *B. cenocepacia* strain PC184. The titer was expressed as the reciprocal of the
778 highest dilution showing inhibition.
779 *Indicates sugars that inhibited tailocin BceTMilo adsorption. Results are representative of
780 triplicate experiments. Each bar represents the standard deviation.
781
782 **FIG 6** Sensitivity of *P. aeruginosa* LPS mutants to tailocin BceTMilo. (**A**) Activity of serially
783 diluted (1:10) tailocin BceTMilo against: PAO1 (i); PAO1*wbpM* (ii)*;* PAO1*wbpL* (iii)*;*
784 PAO1*rmlC* (iv); or PAO1*algC* (v). (**B**) Circled numbers indicated in Fig. 5(A) correspond to
785 depicted LPS structure with same number indicated in Fig. 5(B). Putative residues involved in
786 BceTMilo adsorption are circled. Abbreviations: Man, mannose; Fuc, fucose; GalN, N-
787 galactosamine; HEP, Mannoheptose; NAG, N-acetylglucosamine; KDO, 2-keto-3
788 deoxyoctulosonic acid. Figure adapted from Kohler *et al.* (2010)
789
790 **FIG 7** Electrophoretic profiles of LPS from *B. cenocepacia* K56-2, J2315, K56-2 and K56-2
791 deletion mutants and complements. LPS prepared as described in Materials and Methods. A total
792 of 1.0 µg LPS was loaded in each lane of a 16.5% polyacrylamide gel in a Tricine-SDS system

and developed by silver staining as described in Materials and Methods. Please note that the white line between lanes 3 and 4 indicates that a lane was removed from the original gel, since data was not presented for the strain in Results.

**FIG 8** Sensitivity of *B. cenocepacia* strain K56-2, J2315, and K56-2 LPS mutants to tailocin BceTMilo. (**A**) Activity of serially diluted (1:10) tailocin BceTMilo against: K56-2 (i); J2315 (ii); K56-2 ΔwaaL (iii); or K56-2 ΔwaaC (iv); K56-2 ΔwaaL complement (v); or K56-2 ΔwaaC complement (vi). (**B**) Circled numbers indicated in Fig. 8A correspond to depicted LPS structure with same numbers indicated in Fig. 8B. Residues involved in BceTMilo adsorption are circled. Abbreviations: OS, oligosaccharide; HEP, L-glycero-D-*manno*-heptose; KO, D-glycero-α-D-talo-oct-2-ulopyranolsylonic acid; KDO, 2-keto-3 deoxyoctulosonic acid; QuiNAc, β-D-QuiNAc. Above structure modified from Ortega *et al.* (2009)

806 **TABLE 1** Bacterial strains and plasmids used in this study

| Strain | Relevant Characteristics | Reference or Source |
|---|---|---|
| ***Burkholderia cenocepacia*** | | |
| BC0425 | Producer of BceTMilo, ET12 epidemic lineage, clinical isolate | Laboratory collection |
| AU10487 | Clinical isolate | Laboratory collection |
| K56-2 | ET12 epidemic lineage, O-antigen$^+$ | Sokol et al.(1999) |
| J2315 | ET12 epidemic lineage, O-antigen$^-$ | Laboratory collection |
| PC184 | Midwest epidemic lineage, O-antigen$^-$ | Laboratory collection |
| BC0425 Δ *recA* | BC0425, deleted *recA* gene | This study |
| K56-2 Δ *waaL* | K56-2, deleted *waaL* gene, O-antigen$^-$ L-Rham$^-$ | This study |
| K56-2Δ *waaC* | K56-2, deleted *waaC* gene, O-antigen$^-$ L-Rham$^-$ Glu$^-$ | This study |
| ***Escherichia coli*** | | |
| E. cloni ® 5-alpha | *fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | Lucigen |
| ***Pseudomonas aeruginosa*** | | |
| PAO1 | LPS A$^+$ LPS B$^+$, R2 pyocin producer | J. S. Lam (Burrows *et al.*, 1996) |
| PAO1*wbpM* | LPS A$^+$ LPS B$^-$ | J. S. Lam (Burrows *et al.*, 1996) |
| PAO1*wbpL* | LPS A$^-$ LPS B$^-$ | J. S. Lam (Rocchetta *et al.*, 1998b) |
| PAO1*rmlC* | LPS A$^-$ LPS B$^-$ L-Rham$^-$ | J. S. Lam (Rocchetta *et al.*, 1998a) |
| PAO1*algC* | LPS A$^-$ LPS B$^-$ L-Rham$^-$ Glu$^-$ | J. S. Lam (Jarrell *et al.*, 1977) |
| NIH-K | R1 pyocin producer | ATCC25350 |
| NIH-1 | R5 pyocin producer | ATCC25313 |
| **Plasmids** | | |
| pRK2013 | Tra$^+$ Mob$^+$ ColE1 replicon Km$^r$ | Laboratory Stock |
| pMo130 | Suicide vector for allelic exchange in *Burkholderia*; ColE1 *ori* RK2 *oriT xylE sacB* Km$^r$ | M. I. Voskuil (Hamad *et al.*, 2009) |
| pMo168 | Replicative vector for *Burkholderia*; *ori*pBBR1 *mob*$^+$,*xylE* Km$^R$ | M. I. Voskuil (Hamad *et al.*, 2009) |
| pMo130:: *recA*-UD | pM0130 with *recA* upstream and downstream fragments | This study |
| pMo130:: *waaL*-UD | pM0130 with *waaL* upstream and downstream fragments | This study |
| pmo130:: *waaC*-UD | pM0130 with *waaC* upstream and | This study |

| | | |
|---|---|---|
| | downstream fragments | |
| pMo168:: *recA*-comp | *recA* gene cloned into pMo168 | This study |
| pMo168:: *waaL*-comp | *waaL* gene cloned into pMo168 | This study |
| pMo168:: *waaC*-comp | *waaC* gene cloned into pMo168 | This study |

$Km^r$: resistance to kanamycin.

807
808

809 **TABLE 2** Primers used in this study

| Primer | Relevant Characteristics |
|---|---|
| *recA*-up-F | 5'-GTGGCTAGCTGACCGGATGGATCTGGCGCG-3' |
| *recA*-up-R | 5'-CTCAGATCTCAGCGTCTGTTGGAGGCGCGC-3' |
| *recA*-down-F | 5'-GTGAGATCTGAGTGATGGTTGCGCGCCGAG-3' |
| *recA*-down-R | 5'-GTGAAGCTTACGGGCTCGGATAGCGGCATG-3' |
| *waaL*-up-F | 5'-GTGCGGCCGCTCGCTTCGCCGAGCACCATG-3' |
| *waaL*-up-R | 5'-GTGGGATCCTCTGGGCATTTGCCGGGCTGG-3' |
| *waaL*-down-F | 5'-GTCGGATCCAAGACGCTCCATACGCGCCG-3' |
| *waaL*-down-R | 5'-GACTTCTAGAGCGCACCATCTCCTGCTCGG-3' |
| *waaC*-up-F | 5'-CTGGCTAGCCCCGGGTATTGCGTCGAA-3' |
| *waaC*-up-R | 5'-GTCAGATCTCCTACTGGTCGCCGAACGTCGT-3' |
| *waaC*-down-F | 5'-GTCAGATCTCAGCCGGCGACAGACATAAAG-3' |
| *waaC*-down-R | 5'-CGTAAGCTTCAATCGACGTCGCGGATCAGT-3' |
| *recA*-comp-F | 5'-CTCGGATCCGGAAGATAGCAAGAAGGGCTC-3' |
| *recA*-comp-R | 5'-CTCTCTAGACTCGGCGCGCAACCATCACTC -3' |
| *waa*L-comp-F | 5'-GTGGATCCCATGAGCGGGCTGTCGGTGG-3' |
| *waaL*-comp-R | 5'-CACTTCTAGACAGCAATCGCACGGGCTTGC-3' |
| *waaC*-comp-F | 5'-CACCTGCAGTCGGTTCGCGTGTGGACAGC-3' |
| *waaC*-comp-R | 5'-GACGGATCCTATGTCTGTCGCCGGCTGCC-3' |

810 Added restriction site is underlined.

811

812 **TABLE 3** Tailocin BceTMilo efficiency of killing

| Tail major proteins | Protein Mass (ng/10µl) | Protein Molecules[a] $10^{14}$ per ml | Tailocin Particles[b] $10^{11}$ per ml | KU[c] $10^{11}$ per ml | *EOK*[d] |
|---|---|---|---|---|---|
| Sheath (43 kDa) | 91 | 1.3 | 6.27 | 3.89 | 0.6 |
| | 144 | 2.0 | 9.89 | 3.89 | 0.4 |
| | 151 | 2.1 | 10.40 | 12.30 | 1.2 |
| | 473 | 6.6 | 32.40 | 12.30 | 0.4 |
| Tube (17 kDa) | 36 | 1.3 | 6.20 | 3.89 | 0.6 |
| | 94 | 3.3 | 16.30 | 3.89 | 0.2 |
| | 322 | 11.0 | 55.80 | 12.30 | 0.2 |
| Avg | | | | | 0.5 |

813 [a] Protein Molecules = ((Protein Mass X 1E-9) X 100/ Molecular Mass) X Avogadro's Number
814 [b] Tailocin Particles = Protein molecules/204[e]
815 [c] KU = Killing Unit
816 [d] *EOK* = KU/Tailocin Particle
817 e = Protein Molecules (tube or sheath)/Tailocin

818

819 **TABLE 4** Burst size of tailocin BceTMilo[a]

| | Total Cell No. ($10^9$) | Total KU[b] ($10^{12}$) | KU/Cell |
|---|---|---|---|
| Exp 1 | 7.1 | 4.73 | 660 |
| Exp 2 | 3.5 | 2.14 | 610 |
| Exp 3 | 6.0 | 3.49 | 580 |
| Avg | | | 620 |

820 [a] Burst size was calculated as total KU of lysates divided by total
821 Cell No. exposed to UV.
822 [b] KU = Killing Unit